ON PIGMENT CHANGES AND GROWTH IN THE BLUE-GREEN ALGA, PLECTONEMA NOSTOCORUM BORNET EX GOMONT

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Changes in pigmentation in the algae, as influenced by environmental conditions, have evoked considerable interest in the past and at present. It is the purpose of this paper to describe one of the lesser known instances in which a specific change in pigmentation follows a specific change in the environment of an alga, and some of the practical considerations that may be drawn therefrom.

The theory of chromatic adaptation in the algae commenced in the late 19th century with the observation that there was a relationship between color and depth of zone of growth of marine algae, and has had a controversial course since. Rabinowitch (1945) and Boresch (1932) may be consulted for an extended discussion. Several points have gradually become apparent.

One of the lesser publicized of these has been the observation that in certain blue-green algae, color may vary with nutrition in a dramatic fashion. Apparently the first such observation was made by Boresch (1910) with an unidentified member of the Oscillatoriaceae. He found that after a protracted period of culture, the alga lost its characteristic blue-green color and took on the light brown to golden color of a diatom. Experimentation showed that it was not the wave-length of light, but rather the depletion of nitrogen from the medium, and that alone, which was responsible for the color shift. In this and further work (Boresch, 1913; Magnus and Schindler, 1912; Schindler, 1913), all with members of the Oscillatoriaceae and especially species of *Oscillatoria* and *Phormidium,* it was determined that the color shift was due to a selective disappearance of chlorophyll and phycocyanin, thereby "unmasking" the carotenoids; that a yellowed culture could be made to return to green, sometimes within 24 hours, upon the addition of minute amounts of nitrogen [as little as "1/20,000 normal" KNO_3 (Boresch, 1910) (0.00425 grams per liter)] as nitrate or ammonium ions or various organic nitrogen sources; and that greening would take place in the absence of light. This work was done in the period of active debate concerning the validity of theories of chromatic adaptation. Light was indirectly involved in these experiments since the greater the availability of light, the sooner the nitrogen content of the medium would be used up, other factors being equal, and the quicker and more markedly the color shift would take place. It was noted that the chromatic adaptation, and color change due to nutrition concepts as related to light were ecologically antipathetic. The former states that color shifts occur to allow the organism to adapt itself for the better use of light received, thus increasing its phototrophic assimilation, while the latter states that phototrophic assimilation is decreased by the color shift because of the disappearance of the primary photosynthetic pigments. Although the green to yellow color shift reduces or eliminates phototrophic assimilation, it was held

nevertheless beneficial to the alga in that it allows the organism to enter a state of dormancy until conditions ameliorate.

Material

In the course of other work a pure culture of a blue-green alga was obtained from a natural collection through the use of ultraviolet irradiation. This organism was identified by Dr. Francis Drouet as *Plectonema nostocorum* Bornet ex Gomont. It is somewhat unusual in its minute dimensions, being composed of sheathed trichomes of undifferentiated cells averaging 1.3 μ in width at the widest point by 1.8 μ in length. The sheath increased the width of the filament to 2.25–3.0 μ. Since no heterocysts, akinetes or other possibly resistant entities are formed by this species, and since it has no powers of morphological modification (except perhaps variation in width of the sheath), adaptive reactions to the environment are necessarily at the physiologic level.

Experimental

Nutritionally mediated color shift

Plectonema nostocorum had not been in culture long before the color shift was observed in some cultures. Cultures were grown in 250-ml. Erlenmeyer flasks in several liquid media. These were kept under conditions of controlled temperature (22° C. ± 1° C.) and light (40 foot candles, fluorescent source). Actively growing cultures are a brilliant emerald green. If they are kept in the same culture solution over an extended length of time in the light, the culture will, within the space of a few days, undergo a dramatic color shift from brilliant emerald green through yellow-green to a final yellow with slight overtones or orange or brown. In the latter condition, further lapse of time brings about a slow bleaching so that cultures that have been kept for many months assume the color and resemblance of dirty yellowish cotton-wool. Although at first it was assumed that this color shift was brought about by the death of the organism, further experimentation soon established the fact that it was, rather, based on the exhaustion of the mineral solution in which the organism was growing and that the organism even after many months in the new, yellowed condition was still viable. The addition of small amounts of nitrate to the yellow culture caused it to return to the original green at a very rapid rate. The beginnings of the color shift from yellow to green could be observed within about 24 hours and the shift became complete within three days. Several other elements added similarly had no such effect.

Spectrophotometric analyses of growing and dormant (yellow) cultures have been made. In preparation for this examination, living material was suspended in a few milliliters of culture solution and broken up to as small filament fragments as possible in a special small plastic mortar. The resulting suspension was diluted with distilled water to a degree necessary to give an optical density of approximately 0.65 at the wave-length of greatest absorption. The spectrophotometer used to measure the absorption at the various wave-lengths was a Coleman Universal, Model 14, following the procedures suggested by the manufacturer.

The spectral curve so obtained is somewhat flattened by turbidity and cell mate-

rial other than pigments in the sample. It was not possible to break up the filaments entirely, and the resulting sample showed a range of particle size from just visible to the naked eye down to fragments of filaments one or two cells long. The measurements were checked at wave-length intervals of every 100 millimicrons for possible changes in the reading due to settling of the sample. It was found that the greatest effect was a change of only 0.01 units of optical density over the time of the entire assay.

Although no attempt has been made to determine the exact identity of the pigments contained in the cells of this alga, the absorption curve is reproduced (Fig. 1)

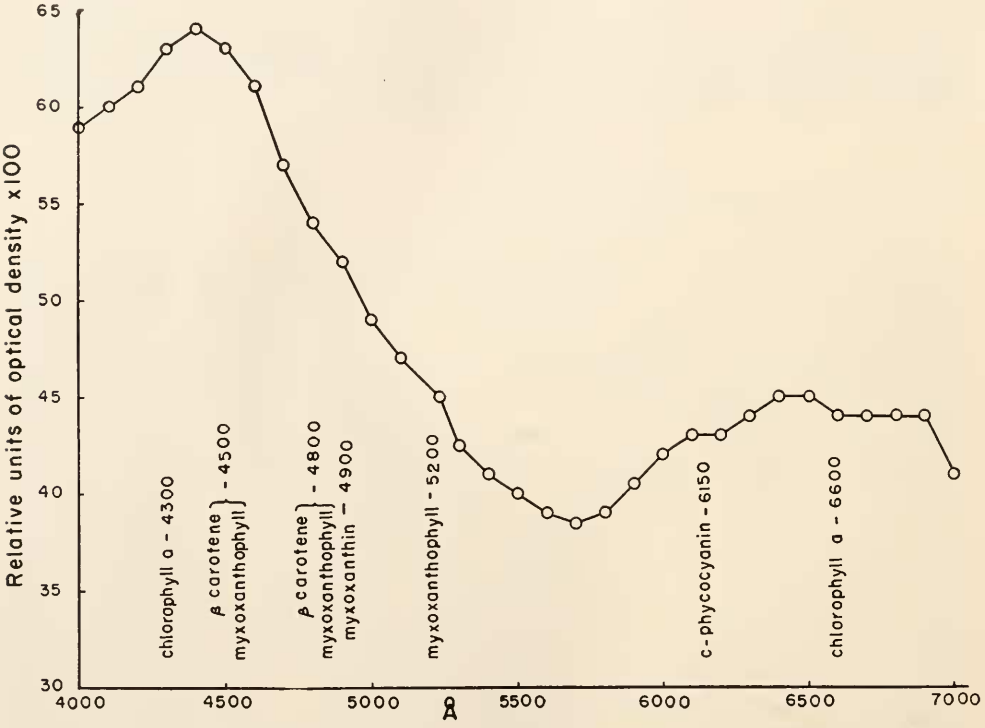

FIGURE 1. Spectrographic analysis of a green, growing culture of *Plectonema nostocorum*.

together with the approximate maximum absorption values for the pigments known to occur in the Cyanophyta (Boresch, 1932; Cook, 1945; Strain, 1951). It will be seen that there is good correspondance between the maxima of pigments generally found in the Cyanophyta and the general shape of the absorption curve for this alga in the active state, except in the region around 640 and 650 millimicrons. Chlorophyll b and c-phycoerythrin are apparently absent in this organism.

Spectrophotometric analyses of the living but yellowed pure cultures were made to determine what pigments had disappeared. As shown in Figure 2, both the chlorophyll and phycocyanin peaks are missing, the major absorption of the cultures now being represented by the presence of carotenoids only.

Growth estimation by pigment analysis

In further exploratory work with this organism it was necessary to develop an accurate method of measuring growth. Because of the small size, filamentous nature, and slow rate of growth of the organism, several of the more usual methods of measuring growth were found to be inadequate. Attention was then given to measurement of growth by means of pigment extraction and its quantitative determination.

Growth is an elusive concept and while it is recognized that pigment measurements do not necessarily precisely indicate the amount of organic matter fixed in the organism because of superimposed ecological variations, on the other hand, if

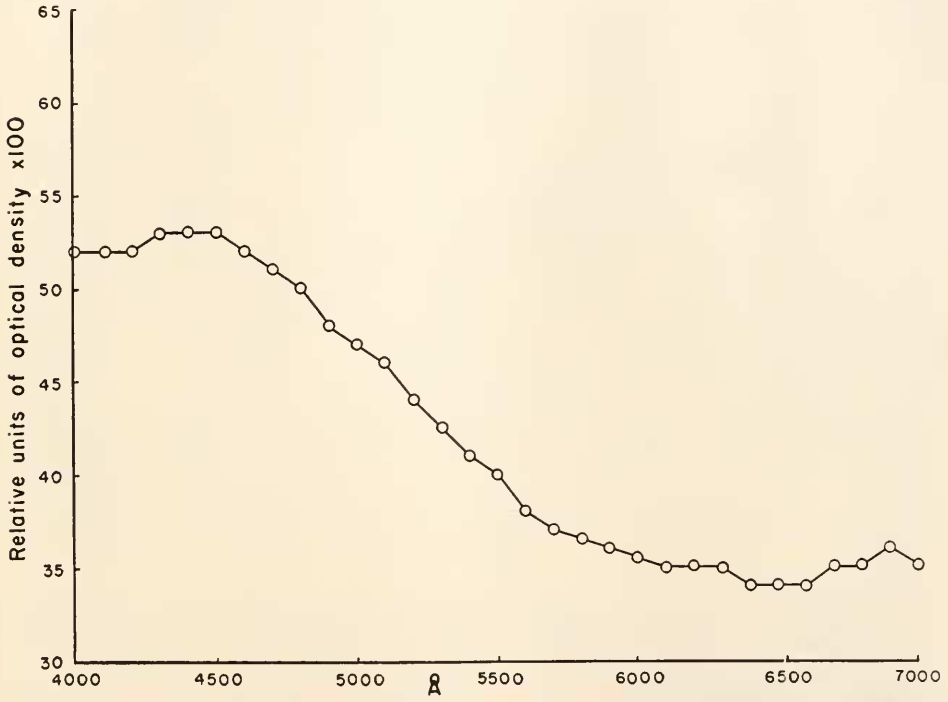

FIGURE 2. Spectrographic analysis of a yellowed culture of *Plectonema nostocorum*.

one allows a more dynamic concept of growth such as total current organic matter-producing potential, then pigment measurement assumes a greater significance (Harvey, 1950). Careful pigment analysis can also be used to characterize and estimate plankton populations (Richards, 1952).

The first to use a quantitative pigment measurement for determination of growth was Harvey (1934) who extracted (with 80% acetone) the pigment from marine plankton collected by net from a known volume of water, and compared this extract visually with calibrated dilutions of a colored standard. This method proved satisfactory for Harvey's work which was with natural populations consisting largely of diatoms. Later Riley (1938) attempted to apply the method to the

measurement of fresh water plankton populations but found that these had a too great and too variable carotenoid content. He was able to adapt the method to his needs by first extracting the chlorophyll from the total pigment complement and measuring it alone visually against calibrated standards. Gardiner (1943) determined, however, through microscopic examination of extracted sample residue, that under these conditions, not all species give up their pigments to the solvent. Further critical work was done by Tucker (1949) who compared results as determined by Harvey's method with actual counts. He found that the correlation in natural populations was not close, and it was his conclusion that it is impossible to get a fixed plankton count as equal to a particular pigment density. This is corroborated in more recent work by Berardi and Tonolli (1953) and Ramamurthy (1953). This might have been expected because of the diverse nature of plankton, containing, as it does, representatives of a wide range of plant groups and an even wider range of animal life, its relative composition depending upon numerous factors such as season, latitude, mineral composition of the waters and others. Comparisons of pigment analyses after Harvey with dry weight determinations by Margalef (1954) have also given a poor correlation even when uni-algal cultures were used. Variation in dry weight per one Harvey and correlated unit of pigment was greater than one power of ten with variations in several factors of culture.

Since none of the above listed difficulties ensues if pigment extractions are made from a pure culture of a pigment-producing organism which has been cultivated under carefully controlled conditions, it might be assumed that the amount of pigment so extracted, measured photoelectrically, would correlate closely with the amount of growth as measured by any other reliable method, especially if the chosen pigment was closely associated with the basic physiology of the cell as are the photosynthetic pigments in autotrophic organisms. This has been borne out in at least one instance. Tucker (1949) found that the coefficient of correlation between the number of cells per unit volume in a pure culture of *Ankistrodesmus,* as measured at various times during the course of its growth, and the amount of chlorophyll per unit volume as sampled at the same times, was 9.97 which represents very close agreement.

On the basis of the above information an attempt was made to establish a workable method of determining growth of the alga through the use of photoelectric measurements of pigment extractions. The immediate problem developing was the decision of which pigment to extract and measure or whether it would be more desirable to measure all pigments at once. Because, as previously described, when the nitrogen composition of the medium becomes sufficiently reduced, the alga loses its green pigments and phycobilins and turns yellow, any method to be fully satisfactory should allow the quantity of growth to be accurately measured whether the culture is in the yellow or green condition. Thus extraction must be done in such a way as to obtain only the yellow pigments which are always present during the growth of the alga and remain after growth ceases.

Extractions of cultures in both the green and yellow conditions were made with various solvents such as acetone, petroleum ether, and methyl alcohol and the extracts were analyzed for pigment complement with the Coleman spectrophotometer as previously described. The effect of the use of heat to destroy enzyme activity quickly and thus prevent degenerative pigment changes was also investigated as

opposed to extractions without this previous treatment. It was found that extractions of green cultures without heat and with 80% aqueous acetone yielded the same pigments as were obtained from yellow cultures treated similarly. The phycobilins are water-soluble and are not extracted from the cells by this method. On the other hand the chlorophyll is apparently destroyed in the extraction. It is quite apparent to the eye that as the acetone is added to the culture, there is a definite and rapid yellowing both of the solution and the filaments themselves. Although acetone solution is a normal procedure for the successful extraction of chlorophyll, it is probable that in this case the chlorophyll is destroyed by reason of the pH at

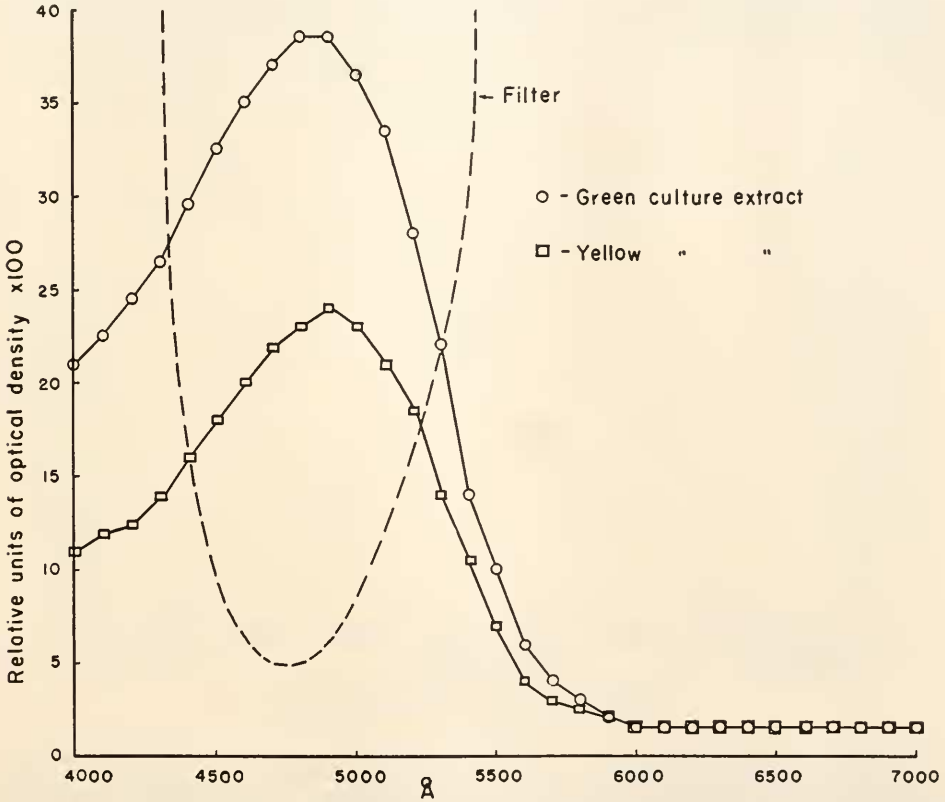

FIGURE 3. Spectrographic analysis of acetone extracts of green and yellow cultures of *Plectonema nostocorum*.

which the extraction is done. Chlorophyll is stable only near neutrality or at a slightly acid reaction. Extraction in this instance is from a culture which has been grown at a pH of 10. Although the culture solution is removed before extraction, the filaments presumedly remain basic. In any case, that the chlorophyll is destroyed and does not appear in the solvent may be seen in the spectrographic photometric analysis of the latter. Figure 3 represents such an analysis in which the curves for 80% acetone extractions of both green and yellow cultures are

plotted. It will be seen that the absorption curves of these are similar, and it is presumed that the pigments are the same. The fact that the absolute amount of pigment is different is not significant since not only were the cultures grown under different conditions for different lengths of time, but also different dilution factors were used in their preparation for analysis.

It should be possible, therefore, to determine the amount of growth in a culture, whether the latter be yellow or green, through a photoelectric determination of the amount of pigment contained in an 80% acetone extract of the culture, other conditions being controlled and equal. A filter was obtained, for use in the Klett-Summerson colorimeter, whose transmittance characteristics resembled the absorption curve of the pigment extract. The transmittance curve for the filter, Wrattan (Eastman Kodak Company) gelatin filter number 45, is reproduced in Figure 3.

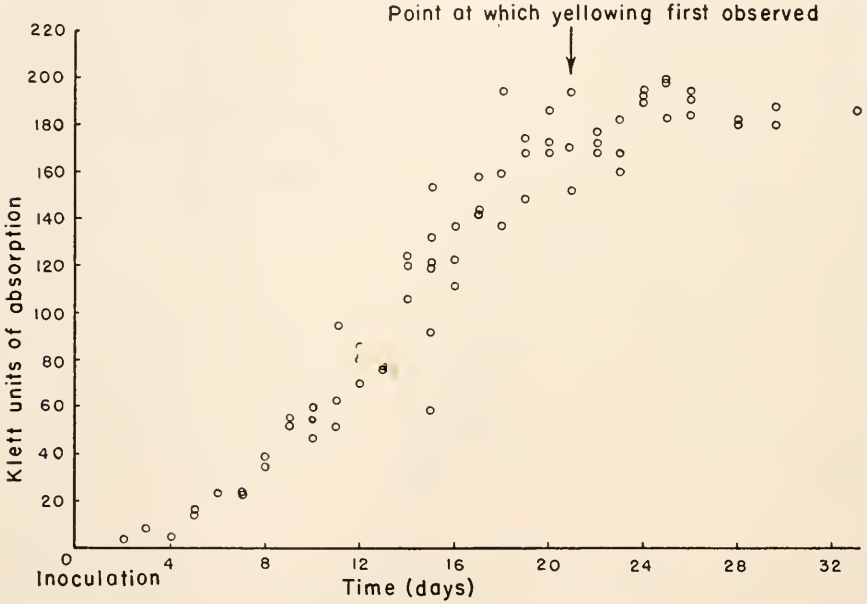

FIGURE 4. Plot of raw growth data.

Three of these filters in series equaled the transmittance of a glass filter made expressly for use in the Klett-Summerson instrument. It was further determined that the heat developed at the surface of the filter was not great enough to damage the gelatin, at least over the length of a normal pigment analysis.

A series of 250-ml. Erlenmeyer flasks was prepared. The nutrient solution was made up with CP grade reagents according to Chu's No. 10 formula (Chu, 1942) in glass-redistilled water and Chu's micrometabolic elements (Chu, 1942) made up similarly were added. The pH was adjusted to 9.7. One hundred ml. of nutrient solution were placed in each of the flasks and autoclaved. Inoculation of all flasks was from the same culture at the same time. One milliliter of this culture, which had been homogenized as much as possible in the Waring Blendor, and which had previously been tested for purity from contamination by inoculation into meat extract

broth, was aseptically introduced into each of the flasks. These were then incubated under constant conditions of light and temperature.

Starting two days from the date of inoculation, one or more cultures were removed daily and assayed in the following manner. The culture solution was filtered through a No. 1, 4.25-cm. Whatman filter disc. The filter paper was then placed in the flask, and the algal material was extracted with 80% aqueous acetone for one hour. The extract was then filtered into a test tube and compared with an 80% acetone blank for pigment absorption using the filter described above in the Klett-Summerson instrument.

The raw data are plotted in Figure 4. It should be noted that the number of cultures removed per day varied from one to six. It should also be noted that the units of absorption are arbitrary. The scale of the instrument is plotted directly in

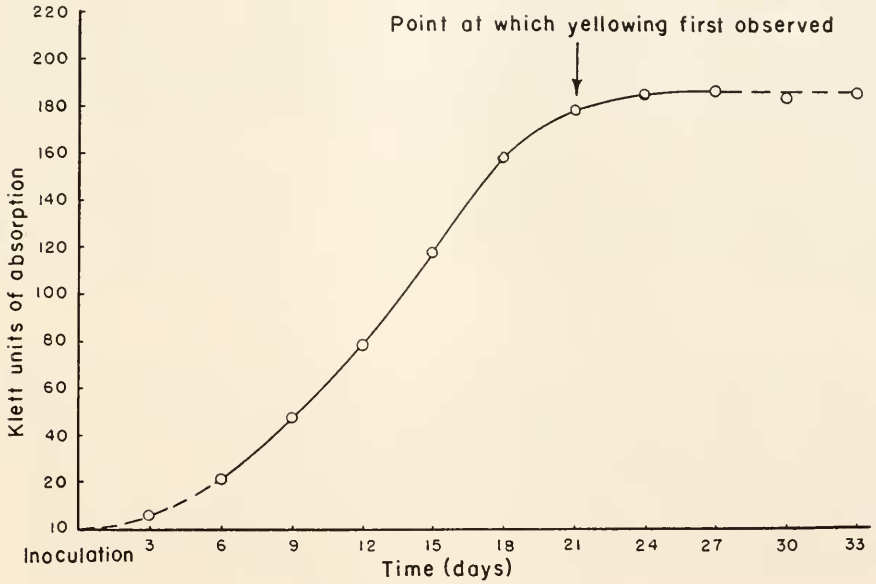

FIGURE 5. Plot of averaged growth data.

a logarithmic fashion; thus the figures are directly proportional to the concentration of the pigment being read (Lambert-Beer Law). A calibration factor, determined by reference to a known standard, may be introduced so that the final readings may be expressed in units of defined value. This was not done. Precautions were taken to insure the flasks being assayed at random and to prevent any unconscious selection on the basis of color.

The following discussion is based upon the curve reproduced in Figure 5. This curve was prepared by grouping the raw data in groups of three days, each day being given equal value, and plotting the mean of the resulting groups. It must be noted that different numbers of cultures were assayed on different days, so that there is not the same degree of statistical reliability throughout the entire curve. The greatest reliability attaches to the central part of the curve.

The point at which yellowing was first observed in the cultures by eye is noted in Figure 5. It will be seen that there is no drop in the curve at this point. It can be expected that there will be a slow decay in the curve from this point, or shortly thereafter, onward through the gradual depauperation of the culture.

As has been stated, it has proven impossible to relate the figures obtained by this method with any other criterion of growth attempted. Dry weight determinations of parallel cultures were so small as to always fall within the limits of possible experimental error until the cultures were half grown. This argues for the sensitivity of the pigment method.

Discussion

Since both chlorophyll and phycocyanin molecules contain considerable amounts of nitrogen, and since the presence or absence of nitrates from the culture medium mediates color shift, it is concluded that the alga is able, when nitrogen becomes unavailable to it from the medium, not only to cease active repairing and construction of nitrogen-containing pigments, but to remove nitrogen actively from already constituted pigment molecules, a conclusion based on the very short time in which the color shift from green to yellow takes place.

Because of the rapidity with which greening takes place in this organism, it is possible to effectively separate pigment synthesis from other growth phenomena.

In this study, pigment evaluation was used as an index of growth. Despite the considerable experimental evidence of others to the inadequacy of this procedure under different conditions, the method appears to have been successful in this instance.

Summary

1. Spectrographic analyses of cultures of *Plectonema nostocorum* are reported, which demonstrate that the nitrogen-containing pigments disappear in the living alga when nitrogen becomes depleted in the culture medium.

2. Re-greening of cultures upon the addition of nitrogen is rapid and can serve to separate the process of pigment synthesis from other processes of growth.

3. Extraction and measurement of the carotenoid pigments at any stage in growth under controlled conditions is considered a reliable index of growth.

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